CHAPTER 5

ENDOTHELIAL PROGENITOR CELLS GIVE RISE TO PRO-ANGIOGENIC SMOOTH MUSCLE-LIKE PROGENY

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ABSTRACT

Reciprocal plasticity exists between endothelial and mesenchymal lineages. For instance, mature endothelial cells adopt a smooth muscle-like phenotype through transforming growth factor beta-1 (TGFβ1)-driven endothelial-to-mesenchymal transdifferentiation (EndMT). Peripheral blood contains circulating endothelial progenitor cells of which the endothelial colony-forming cells (ECFC) harbour stem cell-like properties. Given the plasticity between endothelial and mesenchymal lineages and the stem cell-like properties of ECFC, we hypothesized that ECFC can give rise to smooth muscle-like progeny.

ECFC were stimulated with TGFβ1, after which TGFβ signalling cascades and their downstream effects were investigated. Indeed, EndMT of ECFC resulted in smooth muscle-like progeniture. TGFβ1-driven EndMT is mediated by ALK5 kinase activity, increased downstream Smad2 signalling, and reduced protein levels of inhibitor of DNA-binding protein 3. ECFC lost expression of endothelial markers and endothelial anti-thrombogenic function. Simultaneously, mesenchymal marker expression was gained, cytoskeletal rearrangements occurred, and cells acquired a contractile phenotype. Transdifferentiated ECFC were phenotypically stable and self-sustaining and, importantly, showed fibroblast growth factor-2 and angiopoietin-1-mediated pro-angiogenic paracrine properties.

Our study is the first to demonstrate that ECFC can give rise to smooth muscle-like progeny, with potential therapeutic benefits. These findings further illustrate that ECFCs are highly plastic, which by itself has implications for therapeutical use.

INTRODUCTION

During embryogenesis, endothelial cells arise from hemangioblasts [1]. Smooth muscle cells arise from local mesenchyme and the neural crest [2,3]. As both vascular cell types originate from different sources, it has long been thought that these cells have distinct progenitors. However, in 2000, Yamashita et al. [4] described embryonic vascular progenitor cells that differentiate into both endothelial and smooth muscle cells. These results were later confirmed by Ferreira et al. [5], who showed that endothelial cells and smooth muscle cells, derived from a single embryonic progenitor, integrate into pre-existing vasculature. Postnatally, such common vascular progenitors have not been described yet. However, reciprocal plasticity between endothelial and mesenchymal lineages has been suggested.

Endothelial-to-mesenchymal transdifferentiation (EndMT) was first described in embryonic development, during the formation of the heart valves [6,7]. Later, EndMT of embryonic and adult endothelial cells was also studied *in vitro*, and was shown to be largely TGFβ dependent [8 – 14]. The postnatal role of EndMT in vivo has long been unclear. Recent evidence supports a role for EndMT in cardiovascular fibrosis [15]

Numerous studies have shown that endothelial progenitor cells (EPC) contribute to neovascularization, either by differentiation into cells of the endothelial lineage and local engraftment into to vasculature [16,17] or by secretion of pro-angiogenic factors [18 – 20] In vitro EPC, termed endothelial colony-forming cells (ECFC), can be cultured from mononuclear cells from umbilical cord and adult blood [21]. These cells, with phenotypical and functional characteristics of endothelial cells, show stem cell-like properties, including selfrenewal, as observed by the ability of clonal expansion and high telomerase activity [22].

The apparent plasticity between endothelial and mesenchymal lineages and the stem cell-like properties of ECFC, led us to hypothesize that ECFC, the archetype *in vitro* generated EPC [21], can give rise to smooth muscle-like progeny.

METHODS

CELL CULTURE

Umbilical cord blood was collected at the Department of Gynaecology, Medical Center Leeuwarden, the Netherlands. Cord blood was isolated directly after normal-term delivery, with informed consent from the parents and according to institutional guidelines and the Declaration of Helsinki. Umbilical cord blood-derived ECFC (UC-ECFCs) were isolated and cultured in endothelial outgrowth medium (EOM) up to passage 4. For further experiments, at least three isolations from independent donors were used. For EndMT, UC-ECFC were cultured in mesenchymal differentiation medium (MDM) as described previously [12]. After 21 days, transdifferentiated cells (UC-EndMT) were cultured in basal medium (BM). When applicable, ALK5 kinase activity was inhibited by addition of 10 mM SB-431542 (Sigma, MA, USA), and platelet-derived

growth factor receptor kinase activity was inhibited by addition of 10 mM AG1295 (Calbiochem, Germany).

PHENOTYPIC CHARACTERIZATION

UC-ECFC and UC-EndMT were phenotyped by flow cytometric analysis. Cells were detached using accutase (PAA Laboratories, Austria) and subsequently stained using fluorophore-conjugated antibodies to human CD31 (5µg/mL; IQ Products, The Netherlands), CD34 (5µg/mL; BD Biosciences, CA), CD105 (5µg/mL), CD144 (2.5µg/mL), VEGF-R2 (5µg/mL), Tie-2 (2.5µg/mL), αSMA (2.5µg/mL), TGFβ-R2 (5µg/mL), and PDGF-β (5µg/mL; all R&D Systems, MN), or unconjugated antibodies to human eNOS (2.5µg/mL; BD Biosciences, CA), von Willebrand Factor (3µg/mL; DakoCytomation, Denmark), thrombomodulin (10µg/mL), SM22α (5µg/mL), SM-MHC2 (5µg/mL), Calponin (5µg/mL; all Abcam, UK), ALK1 (5µg/mL; R&D Systems, MN) and ALK5 (2µg/mL; Santa Cruz Biotechnology, CA). Unlabeled primary antibodies were subsequently stained using ither FITC-conjugated donkey antibody fragments to rabbit IgG (5µg/mL; Jackson ImmunoResearch, PA) or fluoroscein-conjugated rabbit antibodies to mouse IgG (5µg/mL; DakoCytomation, Denmark). For detection of intracellular proteins, cells were fixed using 2% paraformaldehyde and permeabilized using 0.1% Saponin (Sigma-Aldrich, MA) prior to staining procedures. Flow cytometric analysis was performed on a FACSCalibur™ (BD Biosciences, CA). Fluorophore-conjugated, isotype-matched nonsense antibodies, as well as fluoresceinconjugated donkey antibody fragments to rabbit IgG and fluorescein-conjugated rabbit antibodies to mouse IgG served as controls.

For visualization of cytoskeletal organizations, cells were fixed in ice-cold methanol:acetone (1:1), rehydrated and incubated with 0.1µM fluorescein-conjugated phallotoxins (Molecular Probes/Invitrogen, OR) in phosphate buffered saline (PBS) containing 3µM 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, MA) for 30 minutes.

For S100A4A detection, samples were fixed in ice-cold methanol:acetone, rehydrated and subsequently incubated with antibodies against S100A4A (5µg/mL; Abcam, UK), followed by incubation with fluorescein-conjugated donkey antibodies to rabbit IgG (5µg/mL; Jackson ImmunoResearch, PA).

For detection of (phosphorylated) SMAD proteins, cells were fixed using 2% paraformaldehyde and permeabilized using 0.1% Saponin (Sigma-Aldrich, MA) prior to staining procedures. Samples were subsequently incubated with antibodies against SMAD2/3 (2µg/mL) and phosphorylated SMAD1/5/8 (pSMAD1/5/8, 2µg/mL; both from Cell Signaling, MA), phosphorylated SMAD2 (pSMAD2, 2µg/mL; Millipore, MA) and SMAD1/5/8 (4µg/mL, Santa Cruz Biotechnology, CA) overnight and incubated the next day using fluorescein-conjugated donkey antibody fragments to rabbit IgG. Labeled samples were visualized using a Leica LMRXA fluorescent microscope and Leica software.

FUNCTIONAL CHARACTERIZATIONS

Uptake of acetylated low-density lipoprotein (acLDL) and binding of Ulex europaeus (UEA-1) were essentially assessed as described previously.23 Capillary sprout formation by UC-ECFC was assessed on MatriGel™ (BD Biosciences, CA, USA) In short, 10µL of MatriGel™ was solidified in µ-Slide Angiogenesis plates (Ibidi GmbH, Germany). Next, 15 000 UC-ECFC were placed on the MatriGel™ and cultured in endothelial outgrowth medium overnight. Sprout formation was analyzed by microscopic analysis.

Thrombin generation was assessed using a thrombin generation assay (HeamoScan, the Netherlands) as described previously [18].

Gene expression of telomerase reverse transcriptase (hTERT) was analysed by RT–PCR. Total RNA was isolated and reverse transcribed as described below. Primers used were hTERT (sense 5′-TGGATGATTTCTTGTTGGTG-3′; antisense 5′-CTTCCAAACTTGCTGATGA-3′) and GAPDH (sense 5′-CTGCCGTCTAGAAAAACCTG-3′; antisense 5′-GTCCAGGGGTCTTACTCCTT-3′). Amplification was performed on a MyCycler (Bio-Rad, VA) in 96-well plates for 30 cycles. Amplimers were separated by gel electrophoresis in a 2% agarose gel. Activity of telomerase was determined using the TeloTAGGG Assay, kindly provided by Roche Applied Science, the Netherlands, following the manufacturer's protocol.

Gel contraction experiments were performed as described elsewhere [12]. After 20 h of spontaneous contraction, transforming growth factor beta-1 (TGFβ1) (5 ng/mL) was added to the culture medium, and additional contraction was measured after 24 h.

IMMUNOBLOT ANALYSIS

Whole cell lysates (20 mg/lane) were electrophoresed in a 10% nondenaturing polyacrylamide gel, blotted onto nitrocellulose, and incubated overnight with primary antibodies to human pSMAD2 (0.3µg/mL; Millipore, MA), SMAD2/3 (0.3µg/mL), pSMAD1/5/8 (0.2µg/mL; both Cell Signaling, MA), SMAD1/5/8 (1µg/mL), Id2 (1µg/mL), Id3 (1µg/mL; all Santa Cruz Biotechnology, CA), or GAPDH (1µg/mL; AbD Serotec, UK). Alkaline phosphatase-conjugated secondary antibodies and NBT/BCIP (Bio-Rad, VA, USA) were used for detection. Densitometric analysis was performed using ImageJ version 1.41 (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

GENE TRANSCRIPT ANALYSIS

RNA isolation was performed using the RNeasy Mini Kit (Qiagen Inc., CA) according to manufacturer's protocol. Subsequently, 1 µg of total RNA was reverse transcribed using the FirstStrand cDNA synthesis kit (Fermentas UAB, Lithuania) according to manufacturer's instructions. The cDNA-equivalent of 5 ng RNA was used for amplification in 384-well microtiter plates in a TaqMan ABI7900HT cycler (Applied

Biosystems, CA) in a final reaction volume of 10 µL containing 5 µL TaqMan universal PCR Master Mix (Applied Biosystems, CA) and 0.5 µL primer/probe mix. Applied Biosystems *'assay on demand'* primer/probe sets were used to detect amplimers of β-2-Microglobulin (β2M; Hs99999907_m1), SM22α (Hs00162558_m1), Calponin (Hs00154543_m1), ALK1 (Hs00163543_m1), ALK5 (Hs00610319_m1), Collagen type I (Hs00164004_m1), Collagen type 3 (Hs00164103_m1), angiopoietin-1 (Hs00181613_m1), CCL2 (Hs00234140_m1), EGF (Hs00153181_m1), BFGF (Hs00266645_m1), HGF (Hs00300159_m1), IGF-1 (Hs00153126_m1) and VEGFa (Hs00173626_m1). Cycle threshold (C_T) values for individual reactions were determined using ABI Prism SDS 2.2 data processing software (Applied Biosystems, CA). To determine differences in expression, C_T-values were normalized against β_2M-expression using the ΔC_T-method ($\Delta C_{T(gene)} = C_{T(gene)} - C_{T(\beta 2M)}$). To correct for interassay variance, ΔC_T values were normalized against expression levels of an external calibrator ($\Delta\Delta C_{T(gene)} = \Delta C_{T(gene)} - \Delta C_{T(calibrator)}$). Relative expression levels were calculated as $2^{-(\Delta\Delta C_T)}$. All cDNA samples were amplified in triplicate. Differences of a two-fold higher or greater were considered to be biologically relevant.

PARACRINE EFFECTS OF UC-ENMT

Conditioned medium (CM) was obtained by incubating UC-EndMT in BM for 72 h. Capillary sprout formation was performed as described above. CM was used with 10 mg/mL fibroblast growth factor (FGF)-2-neutralizing antibodies and/or 10 mg/mL of recombinant human Tie-2/Fc (both from R&D Systems, UK) or 10 mg/ mL irrelevant IgG. Unconditioned BM or BM supplemented with 20 ng/mL FGF-2 and 200 ng/mL angiopoietin-1 (both from Peprotech, NJ, USA) were used as controls. Number of branching points was manually scored, and the cumulative length of sprouts was analysed using ImageJ version 1.41. UC-EndMT at passage 5 were cultured in basal medium for 72h. The conditioned medium (CM) was filtered using 0.2 µm filters to remove cell debris. Capillary sprout formation was performed as described above. CM was used with- or without 10 µg/mL bFGF neutralizing antibodies or 10 µg/mL of recombinant human Tie-2/Fc (both from R&D systems, UK). Unconditioned basal medium or basal medium supplemented with 200 ng/mL bFGF and 20 ng/mL angiopoietin-1 (both from Peprotech, NJ), were used as controls. The number of branching points were manually scored and the cumulative length of sprouts was analyzed using ImageJ version 1.41.

STATISTICAL ANALYSIS

All experimental data are obtained from at least three independent experiments using at least three isolations from unrelated donors. All data are expressed as mean+standard error of mean. For multiple comparisons testing, one-way ANOVA followed by Tukey post hoc analyses was performed. P-values <0.05 were considered to be statistically significant.

RESULTS

CHARACTERIZATION OF ECFC (UC-ECFC)

The first UC-ECFC colonies were observed 9–21 days after plating the initial MNC fraction (Figure 1A), and the cultures reached confluence within the next 5–8 days (Figure 1B). Cells were cultured up to an additional three passages after which EC characteristics were determined by binding of fluorescein-conjugated lectin from Ulex europaeus and uptake of DiI-acLDL (Figure 1C and D, respectively). The ability of UC-ECFC to form capillary-like sprouts on Matrigel was confirmed (Figure 1E). An important functional parameter of EC is their ability to prevent thrombin formation. Indeed, UC-ECFC showed anti-thrombogenic function comparable with human umbilical vein endothelial cells (HUVEC) in an in vitro thrombin generation assay (Figure 1F). UC-ECFC phenotype was further characterized by flow cytometric analysis. Marker proteins of the endothelial cell lineage, namely CD31 (99.68+0.25%), CD144 (94.84+1.31%), VEGFR-2 (97.26+2.06%), eNOS (99.08+0.28%), vWF (99.21+0.75%), thrombomodulin (83.73+9.50%), and Tie-2 (90.77+0.85%) were abundantly expressed by UC-ECFC. In contrast, marker proteins of the mesenchymal cell lineage, namely αSMA (0.82+0.70%), SM22α (2.78+2.00%), SM-MHC2 (3.36+2.13%), and calponin (0.05+ 0.03%), were virtually not expressed by UC-ECFCs. UC-ECFC were further analysed for the expression of CD34 (18.84+1.63%) and for the expression of TGFβ-related growth factor receptors. Abundant expression of ALK1 (99.77+0.06%), CD105 (99.90+ 0.05%), and TGFβ-R2 (78.12+1.42%) was observed on UC-ECFC. In contrast, expression of ALK5 (2.50+0.05%) and PDGF-Rb (4.86+1.92%) was virtually absent (Figure 1G). Gene expression of hTERT was analysed by RT–PCR. UC-ECFC at low-population doublings showed a readily detectable gene expression of hTERT, indicating that these cells are capable of self-renewal (Figure 1H). Telomerase activity of UC-ECFC at lowpopulation doubling numbers (,5) was similar to that of hTERTtransfected HUVEC controls (Figure 1I and J). Telomerase activity reduced with increased population doublings, and was below detection limit after 20 population doublings.

TRANSDIFFERENTIATION OF UC-ECFC TO SMOOTH MUSCLE CELLS

UC-ECFC were cultured up to passage 4 in EOM, after which they were cultured in MDM containing TGFβ1 and PDGF-BB, for an additional 21 days. Transdifferentiated cells (UC-EndMT) had a strong proliferative capacity, and cultures acquired the classical 'hill and valley' morphology (Figure 2A). UC-EndMT had a cytoskeletal organization, typical for smooth muscle-like cells, as shown by staining with phallotoxins (Figure 2B), and expressed the S100 calcium-binding protein A4 (S100A4A) (Figure 2C). EndMT also resulted in changes in functional properties, i.e. gain of contractile phenotype and loss of anti-thrombogenicity (Figure 1F). Cells were embedded in collagen type I gels and cultured for 20 h, which caused a spontaneous contraction of the gel of 30.1+5.9%. In contrast, cell-free controls and gels loaded with UC-ECFC did not contract. Addition of TGFβ1 (5 ng/mL) to these gels for an additional 24 h showed additional contraction

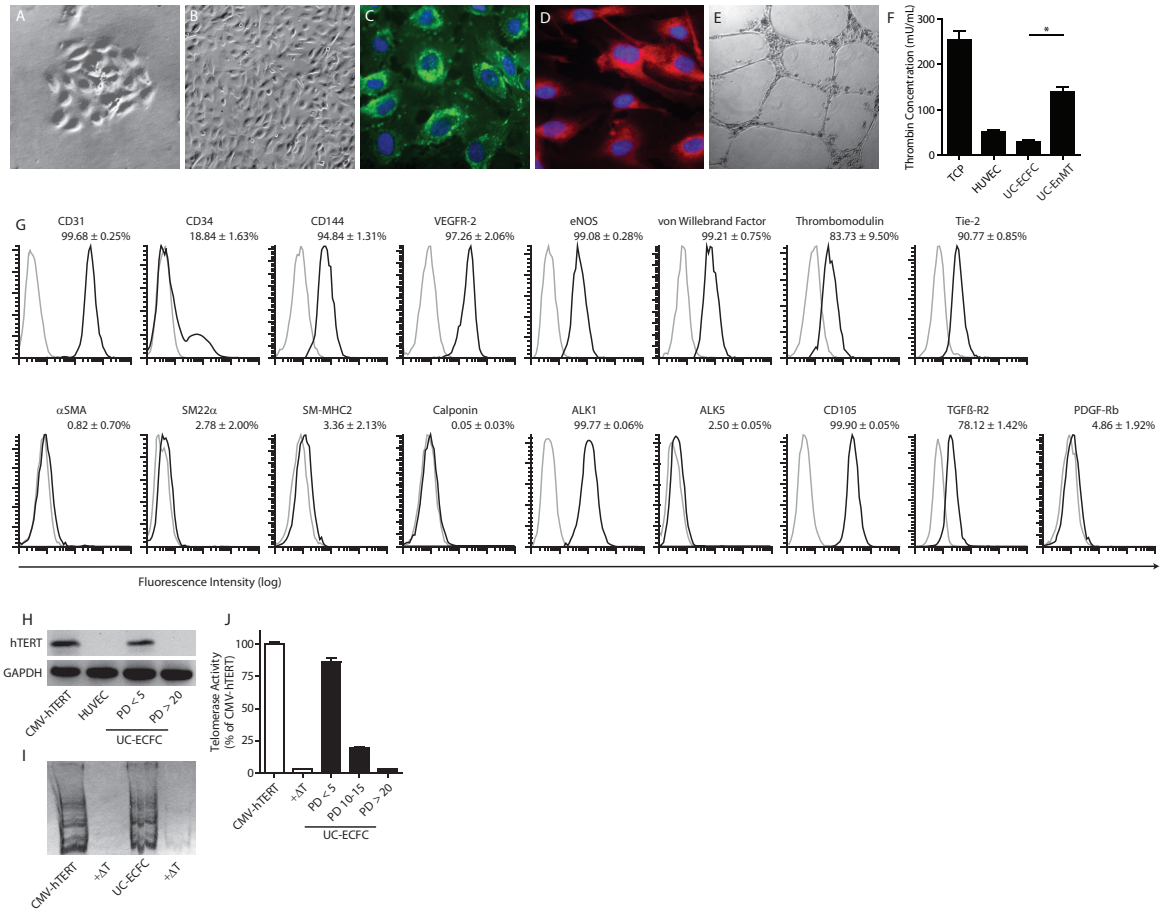

Figure 1. Characterization of umbilical cord blood-derived endothelial outgrowth cells (UC-ECFC). *(A) Cord blood mononuclear cells were cultured in EOM. After 9–21 days, the first outgrowth colonies could be observed. (B) UC-ECFC cultures reached confluency within the next 5–8 days. (C) Binding of FITC-conjugated Ulex europaeus lectin by UC-ECFC was confirmed. (D) Cells took up DiI-acLDL. (E) UC-ECFC formed capillary-like sprouts on Matrigel. (F) Anti-thrombogenic function of UC-ECFC was assessed by measuring their inhibition of thrombin generation. Anti-thrombogenicity of UC-ECFC was comparable with that of HUVEC controls. Transdifferentiated cells showed loss of anti-thrombogenic properties. TCP, tissue culture plate; EndMT, transdifferentiated UC-ECFC; *P<0.05. (G) Flow cytometric analysis of UC-ECFC phenotype. Endothelial markers [(CD31, platelet endothelial cell adhesion molecule-1; CD144, vascular endothelial cadherin; VEGFR-2, vascular endothelial growth factor receptor-2; eNOS, endothelial nitric oxide synthase; von Willebrand factor; thrombomodulin and TEK tyrosine kinase (Tie-2)] were abundantly expressed. Mesenchymal markers (αSMA, alpha smooth muscle actin; SM22α, smooth muscle protein 22 alpha; SM-MHC2, smooth muscle myosin heavy chain 2 and calponin) were virtually absent. High expression levels were found for TGFβ-related growth factor receptors; ALK1, activin like kinase 1; CD105, endoglin; and TGFβ-R2, TGFβ receptor type 2. In contrast to low expression levels of ALK5, activin-like kinase 5 and PDGF-Rb, platelet derived growth factor receptor b. (H) hTERT gene transcript analysis. At higher population doublings, transcript expression was below the detection limit. (I and J) Telomerase activity in UC-ECFC at different population doublings; control is hTERT-immortalized HUVEC (CMV-hTERT). Telomerase activity decreased with increasing population doublings.*

of the transdifferentiated cell-embedded collagen gels to a total of 72.0+2.3% of control, likely due to TGFβ1-induced Rho activation [24], whereas this had no effect on control UC-ECFC embedded gels (Figure 2D and E).

The expression of endothelial lineage markers CD144 (4.06+ 1.83%), eNOS (0.10+0.10%), vWF (0.50+0.45%), thrombomodulin (4.79+0.35%), and Tie-2 (3.54+1.96%) had diminished after transdifferentiation (Figure 2F). About 32% of transdifferentiated

cells (UC-EndMT) still expressed CD31, although the molecular density had decreased by 50-fold. The expression of mesenchymal lineage markers αSMA (96.77+1.43%), SM22α (98.78+1.01%), SMMHC2 (96.30+2.18%), and calponin (90.27+7.09%) was strongly induced during EndMT (Figure 2F).

After 21 days of EndMT, TGFβ1 and PDGF-BB were omitted from the culture media. UC-EndMT were cultured up to an additional seven passages and their mesenchymal phenotype and proliferative behaviour maintained. The expression of endothelial lineage marker CD31 was fully lost after this additional culture period (data not shown). The expression of ALK1 (87.63+1.20%) was reduced after EndMT, whereas the expression of ALK5 (89.08+6.08%) had increased 35-fold compared with UC-ECFC levels, resulting in a shift in ALK1/ALK5 balance (Figure 2F) compared with UC-ECFC (Figure 1F). Furthermore, EndMT resulted in increased expression of PDGF-Rb (38.59+ 6.4).

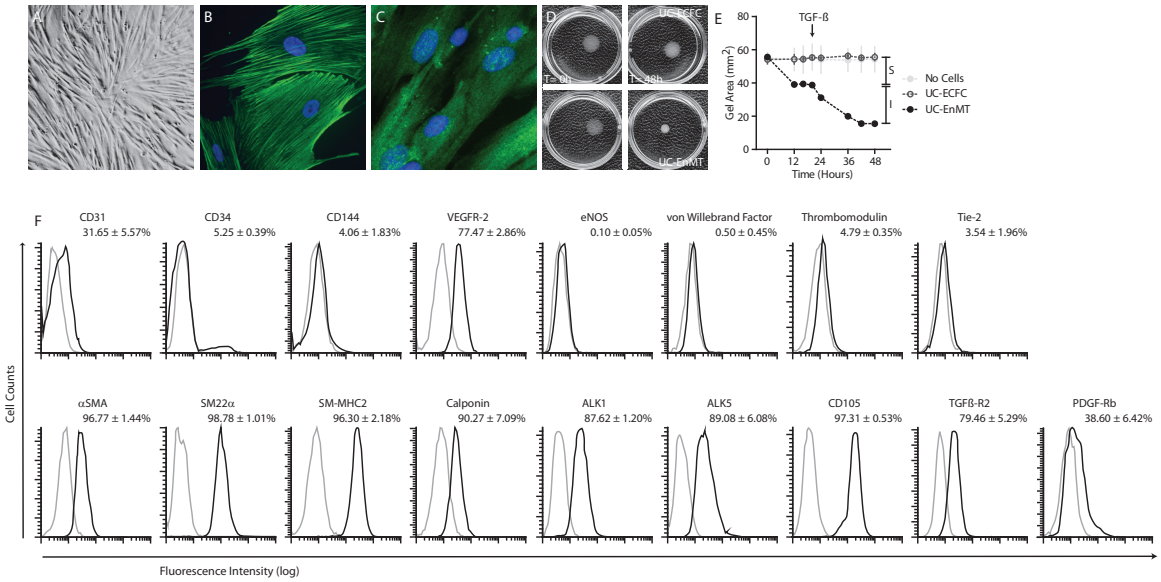

Figure 2. Characterization of transdifferentiated cells (UC-EndMT). *UC-ECFC were cultured in MDM containing TGFβ1 and PDGF-BB, for 21 days after which TGFβ1 and PDGF-BB were omitted from the culture media, and cells were cultured up to an additional seven passages. (A) UC-EndMT showed classical hill-and-valley morphology, characteristic of vascular smooth muscle cells. (B) Staining with FITC-conjugated phallotoxins showed typical cytoskeletal organization. (C) Cells were positive for S100A4A. (D and E) UC-EndMT embedded in collagen type 1 gels for 20 h induced spontaneous contraction (30.1+5.9%) of the gels. UC-ECFC-embedded gels or the cell-free controls did not contract. Additional stimulation with TGFβ1 (5 ng/mL) for 24 h further increased the contraction of UC-EndMT-embedded collagen gels (to a total of 72.0+2.3%). (F) Flow cytometric analysis of UC-EndMT after 21 days of culture in MDM. Expression of endothelial markers (upper row) diminished after transdifferentiation.*
Mesenchymal markers (αSMA, SM22α, SM-MHC2, and calponin) were induced after EndMT. Expression of ALK1 was reduced after EndMT, whereas the expression levels of ALK5 had increased compared with UC-ECFC controls (Figure 1G). Endoglin and TGFβ-R2 expression levels were virtually unchanged after transdifferentiation, whereas expression of PDGF-Rb had increased.

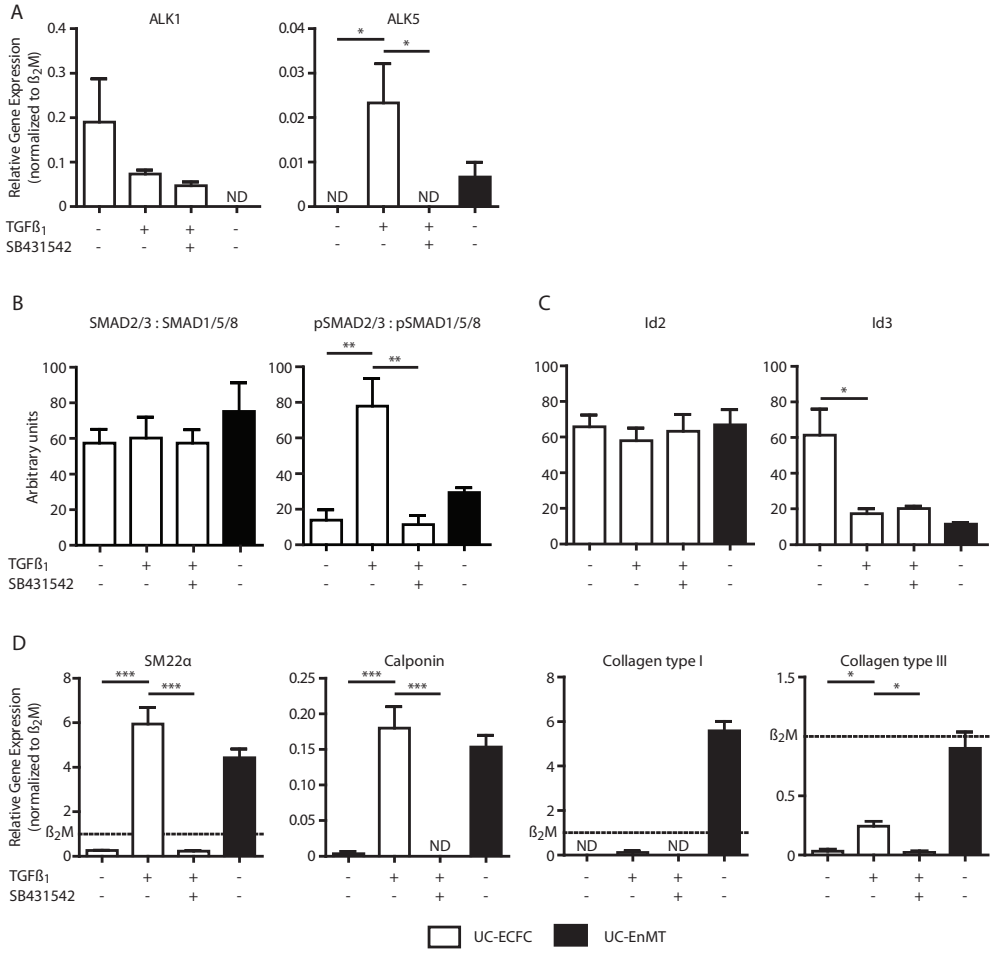

Figure 3. Transdifferentiation of UC-ECFC is mediated by TGFβ1. *EndMT was induced in UC-ECFC by stimulation with TGFβ1 for 96 h. Activinlike kinase 5 (ALK5) inhibitor SB431542 was added to block ALK5-mediated TGFβ signalling. (A) Real-time RT–PCR showed increased gene transcript expression levels of ALK5 after stimulation with TGFβ1. Addition of SB431542 completely inhibited this effect; *P<0.05. (B) Densitometric quantification of SMAD immunoblotting. Protein expression levels of SMAD2/3, SMAD1/5/8, and their phosphorylated forms (pSMAD2 and pSMAD1/5/8) were determined and used to calculate the ratios. Stimulation with TGFβ1 led to increased pSMAD2:pSMAD1/5/8 ratios. Addition of ALK5 kinase inhibitor SB431542 completely abolished these effects; **P , 0.01. (C) Id2 and Id3 are known to antagonize SMAD2/3 signalling by repressing SMAD2-mediated gene transcription.30 The expression levels of Id2 did not change, in contrast to Id3, which showed strongly reduced levels after stimulation with TGFβ1; *P<0.05. (D) Quantitative RT–PCR showed upregulation of SM22α, calponin, and collagen type III. This was completely blocked by SB431542. Expression of collagen type I remained low after 96 h of TGFβ1 stimulation compared with levels of transdifferentiated cells; *P<0.05, ***P<0.001.*

TRANSDIFFERENTIATION OF UC-ECFC IS MEDIATED BY TGFB1

SMAD signalling through the type 1 TGFβ receptors ALK1 and ALK5 was analysed by immunoblotting of the receptor-regulated SMADs, SMAD1/5/8 and SMAD2/3. The ratios were determined between inactive, unphosphorylated SMAD2/3 and SMAD1/5/8 and between the active, phosphorylated forms (pSMAD2:pSMAD1/5/8). Relative gene expression levels of ALK1 and ALK5 and of SMAD target genes were determined. In contrast to UC-ECFCs, UC-EndMT did not express the ALK1 gene. ALK1 is involved in the activation of the SMAD1/5/8 route through phosphorylation

of SMAD1/5/8. The other type 1 TGFβ receptor is ALK5, which antagonizes ALK1 by activation of the SMAD2/3 route [25]. ALK5 expression increased after stimulation with TGFβ1 and completely diminished with addition of its kinase activity inhibitor, SB431542, indicative of a positive feedback system (Figure 3A).

UC-ECFC stimulated with TGFβ1 for 96 h showed increased pSMAD2/3:pSMAD1/5/8 ratios, whereas the ratios between the unphosphorylated forms remained similar (Figure 3B). Addition of the ALK5 kinase inhibitor SB431542 had no effect on basal SMAD2/ 3:SMAD1/5/8 ratios, but normalized the pSMAD2:pSMAD1/5/8 ratios of TGFβ1-stimulated cells to control levels. (Figure 3B). Inhibitors of DNA-binding/differentiation

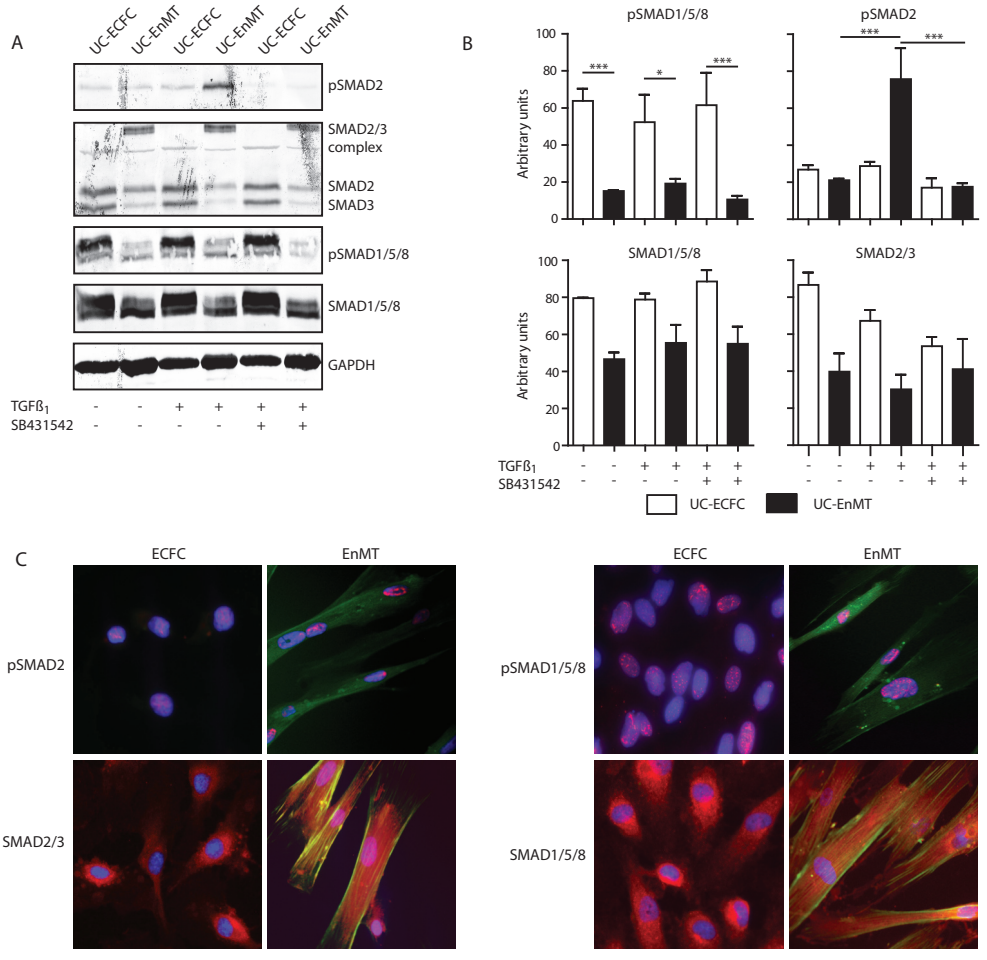

Figure 4. Transdifferentiated cells (UC-EndMT) show increased responsiveness to TGFβ1. *UC-ECFC and UC-EndMT were stimulated with 50 ng/mL TGFβ1 for 1 h. ALK5 kinase inhibitor SB431542 was added to discriminate ALK5-mediated effects of TGFβ1. Immunoblotting was used to study the expression of basal and phosphorylated SMAD1/5/8 and SMAD2/3. (A) Representative images of immunoblotting of TGFβ signalling components SMAD1/5/8, SMAD2/3, and their phosphorylated forms, pSMAD1/5/8 and pSMAD2/3. (B) Densitometric analysis of SMAD immunoblotting. Stimulation of UC-ECFCs and UC-EndMTs with TGFβ1 had no effect on basal SMAD2/3 and SMAD1/5/8 expression. Levels of pSMAD1/5/8 were reduced in UC-EndMT compared with UC-ECFC, irrespective of TGFβ1 stimulation or ALK5 kinase inhibition. TGFβ1 stimulation induced increased expression of pSMAD2 in transdifferentiated cells, which was completely blocked by SB431542. UC-ECFCs did not show increased pSMAD2 levels after stimulation with TGFβ1; *P<0.05, ***P<0.001. (C) Immunofluorescent images showing typical cytoskeletal organization in UC-EndMT by staining with fluorescein-conjugated phallotoxins (green). Unphosphorylated SMAD2/3 and SMAD1/5/8 show cytoplasmic staining patterns (red), whereas their phosphorylated forms (red) show typical nuclear localization (blue).*

proteins (Id proteins) are dominant-negative regulators of basic helix–loop–helix DNA-binding transcriptional regulators which play a role in lineage commitment, cell cycle control, and cell differentiation [26] Expression of Id genes depends on SMAD1/5/8-mediated activation of the Id promoters through binding to SMAD-responsive elements. In contrast, SMAD2/3 signalling inhibits Id gene expression through the activation of the transcriptional repressor ATF3, which binds to ATF/CREB site on the Id promotors and represses transcription [27] In non-transdifferentiated cells, SMAD1/5/8 signalling induces Id3 [28,29], which antagonizes SMAD2/3 signalling by repressing SMAD2-mediated gene transcription [30]. Ectopic expression of Id2 and Id3 has been shown to inhibit transdifferentiation of epithelial cells [31]. Analysis of Id2 and Id3 protein expression in UC-ECFC showed no changes in the expression level of Id2 after TGFβ1 stimulation. In contrast, Id3 was strongly downregulated. This effect was not inhibited by addition of ALK5 kinase inhibitor SB431542 (Figure 3C). Thus, the downregulation of Id3 occurred independently of ALK5 kinase activity.

To study the effect of the altered SMAD and Id signalling during the induction of EndMT, gene expression levels of several mesenchymal genes were studied. Gene expression levels of SM22α and calponin increased after 96 h of stimulation with TGFβ1 and reached levels similar to UC-EndMT (Figure 3D). Inhibition of ALK5 kinase activity with SB431542 decreased pSMAD2:pSMAD1/5/8 ratios and abolished SM22α and calponin gene transcript expression, indicating that both genes are target genes of SMAD2 (Figure 3D). The gene expression level of collagen type I remained low in 96 h of stimulation compared with the expression level in UC-EndMT, in contrast to gene transcription of collagen type III, which had increased. Transcripts of collagen type I and III genes were low compared with UC-EndMT, and diminished after the addition of SB431542 (Figure 3D).

UC-ENDMT SHOW INCREASED RESPONSIVENESS TO TGFB1

UC-ECFC and UC-EndMT were stimulated with 50 ng/mL of TGFβ1 for 1 h with or without the addition of SB431542. Immunoblotting and immunofluorescent imaging was used to study the expression of inactive and phosphorylated SMAD2/3 and SMAD1/5/ 8 (Figure 4A–C). Stimulation of UC-ECFC and UC-EndMT with TGFβ1 had no effect on basal SMAD2/3 and SMAD1/5/8 levels (Figure 4A and B), whereas pSMAD1/5/8 levels were clearly reduced in UC-EndMTs compared with UC-ECFCs. Expression of pSMAD2 was increased in TGFβ1-stimulated transdifferentiated cells and completely diminished with the addition of SB431542. UC-ECFCs did not show increased pSMAD2 levels after short stimulation with TGFβ1 (Figure 4A and B). In contrast to inactive SMAD2/3 and SMAD1/5/8, their phosphorylated forms show nuclear localization (Figure 4C).

PRO-ANGIOGENIC EFFECTS OF UC-ENDMT BY PARACRINE SIGNALLING

To study potential paracrine effects of UC-EndMT on UC-ECFC, we analysed gene transcript levels of several pro-angiogenic factors. Expression levels of HGF, IGF-1,

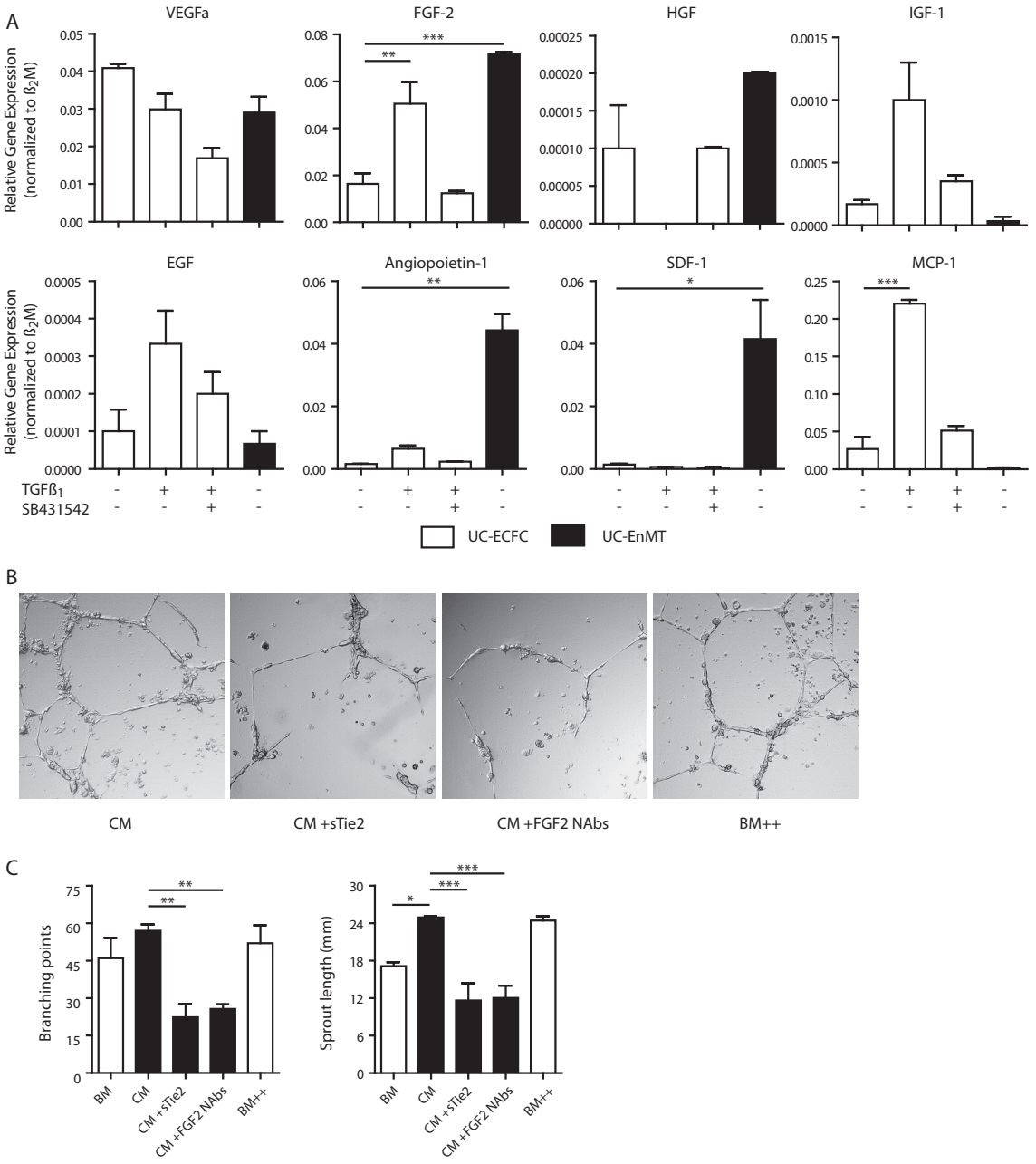

Figure 5. Pro-angiogenic effects of UC-EndMT by paracrine signalling. *(A) Quantitative RT–PCR analysis of gene transcript levels of pro-angiogenic factors normalized to β2M expression. Gene transcript levels of basic FGF-2 and angiopoietin-1 were strongly increased in UC-EndMT compared with UC-ECFC. The increase of FGF-2 expression was induced within 96 h of TGFβ1 stimulation; *P<0.05, **P<0.01, ***P<0.001. Capillary sprouting capacity of UC-ECFC was studied using UC-EndMT CM with 10 mg/mL irrelevant IgG (CM), with 10 mg/mL FGF-2-neutralizing antibodies (FGF2 NAbs), or with 10 mg/mL recombinant human Tie-2/Fc (sTie2). Unconditioned BM or BM supplemented with 200 ng/mL FGF-2 and 20 ng/mL angiopoietin-1 (BM++) were used as controls. The total numbers of branching points were determined and the cumulative length of spouts was analysed. (B) Representative images of capillary sprout formation on Matrigel. Note the reduced capillary formation in sTie2 and FGF2 NAbs-treated cultures. (C) The total number of branching points was similar between BM, BM++, and CM. Numbers were reduced with the addition of FGF2 NAbs or sTie2 to CM. The cumulative sprout length was increased with CM compared with unconditioned BM, similar to that observed with BM++. Addition of FGF2 NAbs or sTie2 completely diminished these effects; *P<0.05, **P<0.01, ***P<0.001.*

and EGF were low in both UC-ECFC and UC-EndMT. Expression levels of VEGFa were similar in UC-ECFC and UC-EndMT. Interestingly, gene transcript levels of FGF-2 and angiopoietin-1 were strongly increased in UC-EndMT compared with UC-ECFCs. In contrast to angiopoietin-1 expression, the increase of FGF-2 expression was induced within 96 h of TGFβ1 stimulation (Figure 5A). SDF-1 and MCP-1 are angiogenic factors which play an important role in the recruitment and differentiation of EPC [20,32 – 34]. UC-EndMT expressed high levels of SDF-1, which were virtually absent in UC-ECFC, despite TGFβ1 stimulation for 96 h. MCP-1 expression, however, was induced by short-term TGFβ1 stimulation and almost absent in (quiescent) UC-EndMT. These findings distinguish short-term effects of TGFβ1 stimulation, namely monocyte recruitment by MCP-1, and long-term effects characterized by increased pro-angiogenic cytokine production and SDF-1-mediated recruitment of EPC. UC-EndMT CM was used to study the paracrine effects of UC-EndMT on capillary sprouting of UC-ECFC. Given the increased gene transcript levels of FGF-2 and angiopoietin-1 found in UC-EndMT, neutralizing antibodies to FGF-2 and soluble Tie-2 (sTie-2) were added to CM. The total number of branching points was comparable between BM, BM with FGF-2 and angiopoietin-1 (BM++), and CM (46+8, 52+7, and 57+3, respectively). Neutralization of FGF-2 or addition of sTie-2 to the culture medium resulted in strongly reduced numbers of branching points (Figure 5B and C). The cumulative sprout length was also determined. CM showed increased cumulative length (45.63+1.18%) compared with unconditioned BM. BM supplemented with FGF-2 and angiopoietin-1 showed a similar increase in cumulative sprout length (42.66+4.04%). Addition of FGF-2-neutralizing antibodies or sTie-2 completely diminished the observed increase, indicating FGF-2- and angiopoietin-1-mediated effects (Figure 5B and C).

DISCUSSION

In the present study, we show the intrinsic capacity of UC-ECFC to transdifferentiate into smooth muscle-like cells in an ALK5-dependent manner. The following are our findings: (i) EndMT of ECFC resulted in the waning of endothelial markers and loss of endothelial functionality; (ii) mesenchymal marker expression and contractile function were gained; (iii) EndMT is mediated by ALK5 kinase activity, and characterized by increased SMAD2 signalling and reduced levels of Id3; (iv) transdifferentiated cells (UC-EndMT) were phenotypically stable, self-sustaining, independent of exogenously added transdifferentiation mediators, and (v) harboured pro-angiogenic paracrine properties. To our knowledge, this study is the first to describe the plasticity of UC-ECFC towards smooth muscle cell differentiation.

Since their first description [35], EPC have been extensively studied, and their availability and angiogenic properties have advocated them as an interesting and promising cell source for therapeutic neovascularization [36]. Despite this large research effort, plasticity of EPC has received little attention. *In vivo*, bone marrow-derived smooth muscle progenitor cells (SMPC) have been shown to play a role in cardiovascular pathology [37,38] SMPC have been cultured from umbilical cord and adult blood, although their origin remains largely unclear [39,40]. Our results indicate that ECFC, the archetype *in vitro* EPC [21], can also give rise to smooth muscle-like

progeny. Transdifferentiated cells (UC-EndMT) expressed S100A4A, a member of the S100 superfamily of calcium-binding proteins, commonly used to identify both myofibroblasts and activated smooth muscle cells [41]. UC-ECFC stimulated with TGFβ1 for 96h showed increased pSMAD2/3:pSMAD1/5/8 ratios, which diminished after ALK5 kinase inhibition. Also, Id3 was strongly downregulated. This effect was not inhibited by ALK5 kinase inhibition. Given the repressive effects of Id3 on SMAD2-mediated gene transcription, its downregulation likely contributed to increased SMAD2 signalling. Thus, TGFβ1-induced transdifferentiation occurred via both ALK5 kinase dependent and -independent mechanisms. PDGF-BB was also shown to induce transdifferentiation through an ALK5-dependent mechanism. Previous studies have indicated that the main mechanism of PDGF-BB signalling in VSMC involves TGFβ and that PDGF-BB stimulation of SMC leads to an acute induction of TGFβ expression via MAPK/ERK pathway [42,43]. Here, PDGF-BB stimulation induced EndMT through PDGF-receptor kinase-independent mechanisms. Whether the same mechanisms are involved in PDGF-BB-mediated induction of transdifferentiation remains object of further studies.

The capacity of ECFC to transdifferentiate to smooth muscle-like cells certainly has implications for their therapeutic use in cardiovascular disease. The majority of patients eligible for cardiovascular cell therapy commonly share an inflammatory vascular profile [44]. TGFβ1 and its downstream effector pSMAD2 have been shown to be highly expressed at atherosclerotic lesion sites [45]. Recent data from Frutkin et al. indicate that overexpression of TGFβ1 can have anti-atherogenic effects [46]. Our data show that increased TGFβ signalling results in mesenchymal differentiation of EPC. This has potential beneficial effects, because transdifferentiated EPCs could help limit plaque formation or increase plaque stability [47]. On the other hand, EndMT of EPC could contribute to atherogenesis in an adverse vascular microenvironment [37]. Co-administration of SMPC and EPC has recently been shown to have synergetic angiogenic effects by increased paracrine signalling, mainly through the angiopoietin/Tie2 signalling pathway [48]. Our results corroborate these findings and show that angiopoietin-1-mediated pro-angiogenic effects can be exerted by transdifferentiated ECFC as well. In addition, we show that similar pro-angiogenic effects were mediated by FGF-2 secreted by UC-EndMT. ECFC EndMT thereby provides a novel therapeutic strategy for treating ischaemic cardiovascular disease. *Ex vivo* priming of ECFC has recently been described to enhance their angiogenic potential [34]. Our results illustrate how *ex vivo* stimulation can direct ECFC differentiation, thereby changing phenotypical and functional properties of the cells. This phenomenon can be used in tuning ECFC, creating tailored therapy for use in a specific patient with specific underlying pathology and co-morbidity.

In conclusion, our study demonstrates that ECFC give rise to smooth muscle-like progeny by ALK5-mediated EndMT. This high plasticity of ECFC clearly has implications for therapeutical use, e.g. by providing opportunities for *ex vivo* intervention prior to administration. Whether the intrinsic plasticity, or capacity of ECFC for EndMT, differs between healthy subjects and those with cardiovascular disease remains to be addressed in future studies.

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